J. Cell Sci. **64**, 1–11 (1983)
Printed in Great Britain © *The Company of Biologists Limited 1983*

INCREASED CONTRACTILE STRENGTH AND TIGHTENED ADHESIONS TO THE SUBSTRATUM RESULT FROM REVERSE TRANSFORMATION OF CHO CELLS BY DIBUTYRYL CYCLIC ADENOSINE MONOPHOSPHATE

W. MARK LEADER, DAVID STOPAK AND ALBERT K. HARRIS*
Department of Biology, Wilson Hall (046A), University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27514, U.S.A.

SUMMARY

We have examined the changes in cellular contractility and adhesive morphology that accompany the reverse transformation of CHO cells caused by dibutyryl cyclic adenosine monophosphate. The ability of these cells to distort elastic silicone rubber substrata was found to increase markedly over the 5 h period following addition of cyclic AMP and testosterone to the medium, and to relax again following hormone removal. Parallel changes were observed in the interference reflexion image of the adhesion of these cells to glass substrata; broad grey-appearing 'close' contacts characteristic of the transformed state were gradually replaced by numerous small black-appearing 'focal contacts'. These changes in adhesive morphology required considerably longer (20–25 h) to be completed or to revert, however.

INTRODUCTION

Neoplastic transformation causes significant morphological changes in the appearance and behaviour of cells. Many of these changes have been studied in tissue culture (Vasiliev & Gelfand, 1981), while others are seen *in vivo*, where they form the basis of the various empirical criteria that pathologists use to diagnose malignancy (Gompel, 1978). If we believe that living cells are shaped by complex balances of mutually opposing forces, then we must expect these morphological peculiarities of the transformed state to be caused by one or more alterations in the physical properties of cellular components. Unfortunately, the nature of these changes is still not understood, nor is it known whether or not there is some one basic alteration of which all of the various behavioural and morphological changes are secondary consequences.

Two particular cellular properties that have been implicated as possible causes (or at least consistent correlates) of transformation to the cancerous state are adhesiveness and contractility. Both the adhesiveness and the contractile strength of transformed cells seem consistently to be weakened relative to normal or untransformed cells of the same type. For example, Coman (1944) found, by using micromanipulation to pull adjacent cells apart from one another, that much stronger forces are needed to separate the cells of normal keratinizing epithelia than are required to separate carcinoma cells.

* Author to whom correspondence should be addressed.

Subsequently it has been found in a cell reaggregation system that transformed fibroblasts will sort out externally to equivalent untransformed fibroblasts (Gershman, Drumm & Culp, 1976). According to the differential adhesion hypothesis this would mean that the adhesiveness of the transformed cells must be less than that of the normal cells, although it has also been proposed that an external position in the cell-sorting hierarchy could result from reduced contractility. Also contributing to the belief that transformed cells have a reduced adhesiveness is the less-complete spreading of these cells in tissue culture. Indeed, it is even possible to mimic the morphology of transformed cells by culturing equivalent normal cells on artificial substrata, which are relatively non-adhesive. This and related phenomena have been discussed by Carter (1968) in relation to the invasiveness of cancerous cells.

The contractile strength of cells, as judged by their ability to distort elastic substrata, has also been found to be weakened in transformed cells. This has now been observed in a variety of cells using elastic substrata consisting of clotted plasma fibrin (Harris, 1973*b*), reprecipitated collagen gels (Steinberg, Smith, Collozo & Pollack, 1980), or silicone rubber (Harris, Wild & Stopak, 1980). It is interesting that transformed cells seem to resemble leucocytes in both their weak contractility and their weak adhesiveness – as well, of course, as in their invasiveness.

In order to study the changes in cell contractility and adhesiveness that accompany transformation, we chose to use the Chinese hamster ovary (CHO) cell system in which one is able to 'reverse transform' the morphology of this transformed line, simply by adding dibutyryl cyclic adenosine monophosphate and testosterone to their culture medium (Hsie & Puck, 1971; Johnson, Friedman & Pastan, 1971). Such reverse transformed cells spread more fully on the culture substratum, their surfaces become more quiescent (Porter, Puck, Hsie & Kelly, 1974), and their cytoplasmic myosin becomes condensed into oriented bundles called stress fibres (Bloom & Lockwood, 1980). The transformed phenotype can also be conveniently turned on and off by using cells transformed by one of the temperature-sensitive mutants of oncogenic viruses (such as simian virus 40), and a parallel study is being carried out using that system.

In order to follow changes in cell adhesion and contractility over a period of time it is necessary to use detection methods that do not kill or even disturb the cells. For this reason, neither micromanipulation nor electron microscopy would have been practical. To observe the cell-to-substratum adhesions non-destructively we used the optical method called interference reflexion microscopy (Curtis, 1964; Izzard & Lochner, 1976). For the purpose of following changes in cell contractility, we cultured the cells on thin layers of silicone rubber (Harris *et al.* 1980) and recorded the changes in substratum wrinkling patterns using time-lapse cinemicrography.

MATERIALS AND METHODS

Cells

The CHO cells were kindly provided by Dr Arthur Lockwood of the University of N. Carolina Department of Anatomy, and were maintained in Falcon plastic tissue-culture flasks at 37 °C in

F-10 nutrient medium supplemented with 10% foetal calf serum and 0·05 mg/ml gentamycin (Schering). The medium was buffered with 20 mM-*N*-2-hydroxyethylpiperazine-*N'*-2'-ethanesulphonic acid (HEPES). The F-10 medium, foetal calf serum and HEPES were all from Grand Island Biological. Subculturing was carried out prior to confluence using gentle trypsinization in the presence of EDTA. Cells to be reverse transformed were plated out at least 18 h prior to use. Reverse transformation was induced by adding *N*6,*O*2'-dibutyryl adenosine 3':5'-cyclic monophosphate (db-cAMP) and testosterone propionate (both from Sigma), the former from a freshly prepared 100× stock solution in distilled water and the latter from a 500× stock solution in 95% ethanol. Final concentrations in the culture medium were 0·40 mM-db-cAMP and 15 μM-testosterone propionate. In those experiments in which reverse transformed cells were permitted to return to their transformed state, the medium containing db-cAMP and testosterone was replaced with conditioned medium from an untreated culture, rather than with fresh medium. This was done so as to avoid confusing the effects of fresh medium with those of the hormone removal. Cultures to be used for interference reflexion microscopy were washed three times in phosphate-buffered saline before adding the conditioned medium, but it was not practical to carry out such a rinse with cells cultured on silicone rubber sheets, because of the extreme fragility of these sheets.

Silicone rubber substrata

Silicone rubber culture substrata were prepared by briefly flaming a thin layer of silicone fluid (poly(dimethyl siloxane), 30 000 centipoise viscosity, manufactured by Dow-Corning, Midland, Michigan, U.S.A. but purchased from Hopkins and Williams Co., Chadwell Heath, Essex, England). The fluid was spread onto ordinary glass coverslips and its surface crosslinked to form a rubber film, as described previously (Harris *et al.* 1980).

Interference reflexion microscopy

The culture substrata used for this method were no. 1-1/2 Corning coverslips, sealed with heated dental wax over 10 mm holes drilled through the bottoms of polystyrene Petri dishes. A Zeiss IM35 inverted microscope was used, the epi-illumination system of which we modified slightly for this technique. A coverslip half-silvered with aluminium was mounted in the rotating reflector housing in place of the fluorescence barrier and excitation filters and mirror. For routine observation and time-lapse cinemicrography of cell-contact patterns we used a Zeiss Plan-Neofluar 25× oil/water-immersion objective and a Planapo 40× oil-immersion objective together with the tungsten light source and with the epi-illumination field diaphragm stopped down to minimize light scattering. For still photography (including Fig. 1) we used a mercury light source and an Antiflex quarter wave plate mounted on a 40× Epiplan LD objective.

Time-lapse films

Cell behaviour on the silicone rubber substrata was recorded by time-lapse cinemicrography both before and after addition of db-cAMP and testosterone, as well as following its replacement with conditioned medium. Films were made using a Wild intervalometer and a Bolex 16 mm movie camera mounted on a Zeiss inverted microscope using a Neofluar 16× objective. In a few cases an Olympus inverted microscope with a 10× objective was used. Kodak Plus-X reversal film was used in all cases.

RESULTS

Cell morphology

CHO cells were able to attach and spread on both glass coverslips and sheets of silicone rubber. In the absence of db-cAMP and testosterone these cells spread only partially on either substratum (Fig. 1A), extending only poorly developed leading lamellae, just as others have described previously (Porter *et al.* 1974). When observed in time-lapse films, the surface movements of the cells in this state could be seen to

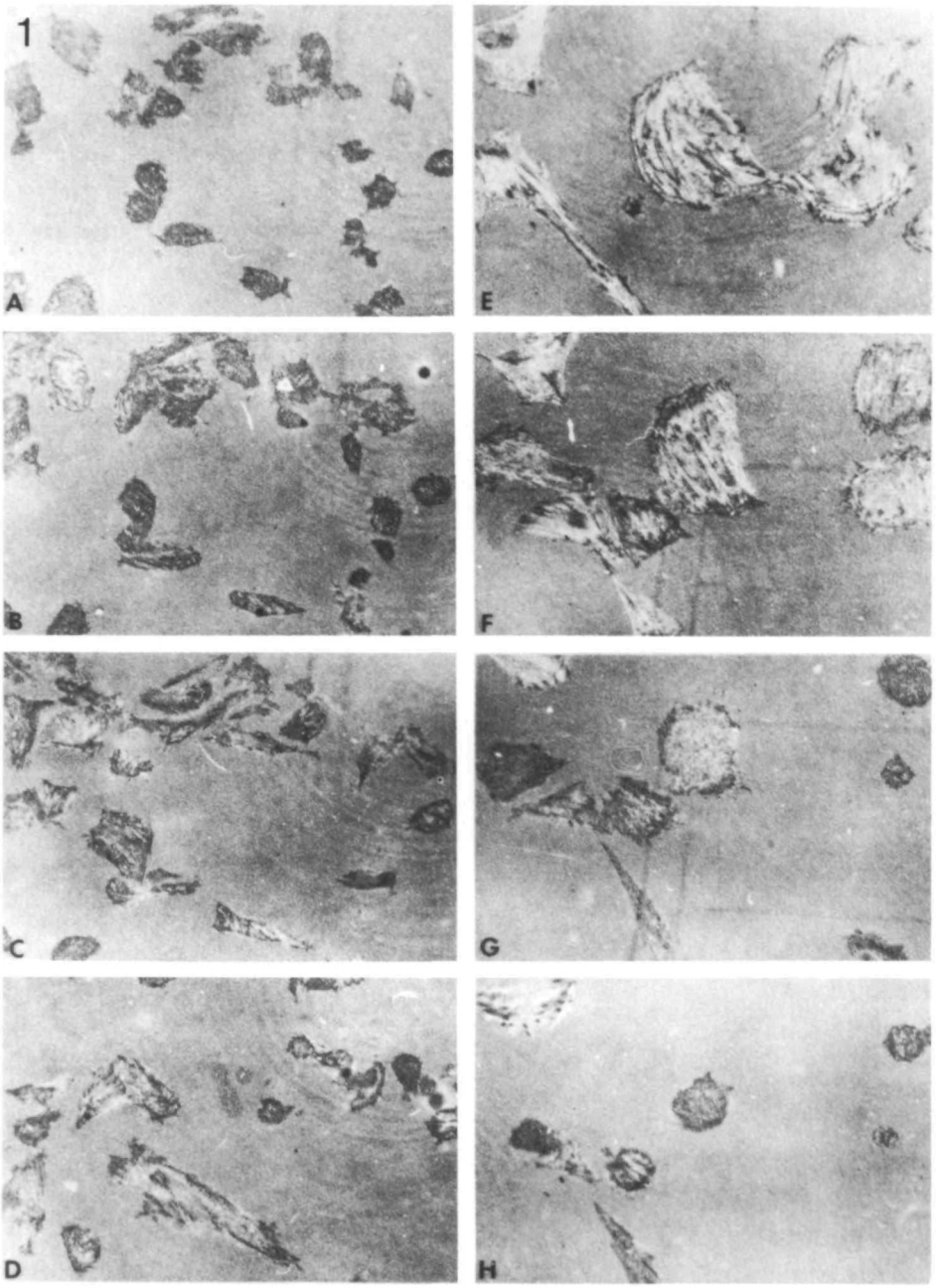

Fig. 1. Development of focal adhesion plaques to glass substrata following reverse transformation of CHO cells by db-cAMP. A single microscopic field of living CHO cells cultured on glass coverslips and photographed by interference reflexion microscopy at sequential times following addition (A–E) and removal (F–H) of db-cAMP. Each field is 190 μm wide. A. Time 0, introduction of db-cAMP; B, after 1 h db-cAMP; C, after 2 h db-cAMP; D, after 8 h db-cAMP; E, after 25 h db-cAMP (db-cAMP removed from this culture at this time); F, 3 h after removal of db-cAMP; G, 12 h after removal of db-cAMP; H, 18 h after removal of db-cAMP.

consist primarily of the repeated protrusion and withdrawal of many small blister-like blebs (Puck, Waldren & Hsie, 1974). Among cells in this transformed state there was only occasional ruffling activity and the cells achieved little or no net translocation, merely blebbing in place.

Following the addition of db-cAMP to the culture medium, a gradual increase in cell spreading occurred. This increased spreading is evident in the interference reflexion images of Fig. 1A–E. The individual cells became progressively more flattened onto the substratum over 10–20 h until visual inspection and comparison of sequential photographs of the same cells in time-lapse films (see also Fig. 1) showed that their spread areas had increased by three to fivefold and occasionally more. In some of these cells, this morphological reverse transformation involves a directional polarization of their spreading, so that cells that had previously been more or less circular became elongate as spreading proceeded. Spreading was complete after 20–24 h and did not continue to increase. During this progressive increase in spreading the blebbing activity of the cell surface was gradually replaced by typical ruffling, that is, repeated back-folding of thin lamellipodia along the cell margins. Even when maximally spread these cells underwent very little net translocation.

When returned to conditioned culture medium lacking db-cAMP, these reverse transformed CHO cells slowly reverted back to their original, poorly spread morphology. This process of morphological reversion was very gradual, like the original reverse transformation requiring 20–25 h to reach completion (a morphology indistinguishable from that of cells that had not been reverse transformed). During this period of reversion, blebbing gradually replaced ruffling along the cell margins.

Interference reflexion microscopy

When cultured without db-cAMP, all but a small minority of CHO cells formed only broad, grey-appearing contacts to the substratum; contacts of the class calculated to have a gap of approximately 30 nm (Izzard & Lochner, 1976). Although the interference reflexion image of these contacts was usually somewhat uneven or blotchy (Fig. 1A), as if the cell-to-substratum distance fluctuated slightly within the contacts, it is noteworthy that these contacts extend so broadly across the lower surfaces of the cells. In particular, note that these contacts even extend directly beneath central areas of the cells, where the nuclei are located. Such a broad and diffuse distribution of adhesive contacts to the substratum is in marked contrast to that previously described for a variety of cultured fibroblasts and other cells (Harris, 1973*a*; Izzard & Lochner, 1976).

A small minority of the cells in these cultures did form the small black-appearing focal contacts, which are normally formed by untransformed fibroblasts and whose dark colour indicates very close contact (10 nm) between the plasma membrane and the substratum. We believe that these images correspond to a revertant, or otherwise not transformed, sub-population among the CHO cells. These aberrant cells were also more fully spread and in general resembled reverse transformed cells.

Following perfusion with culture medium containing db-cAMP and testosterone, the increased spreading of the CHO cells was accompanied by a progressive

replacement of the broad, grey contacts by small black-appearing, focal contacts, or 'adhesion plaques'. Between these small focal contacts, appear broad areas of cell–substratum apposition whose interference reflexion images are white or pastel in colour. These correspond to gap distances of 50–100 nm or greater, and are believed to reflect a lack of contact or adhesion between the cell and its substratum over these areas. The black focal contacts formed primarily along the advancing, outward-spreading parts of the cell margins, while the non-adherent (white or pastel) areas developed for the most part in the central areas and beneath the nuclei. This change in adhesive morphology was already clearly detectable in about half the cells after about 2 h of reverse transformation (Fig. 1B, C) and was well advanced in all cells within 8 h. This progressive concentration or focusing of the adhesive contacts into small and largely peripheral foci was very gradual and did not reach completion until 20–25 h after the introduction of the hormones. By that time, the interference

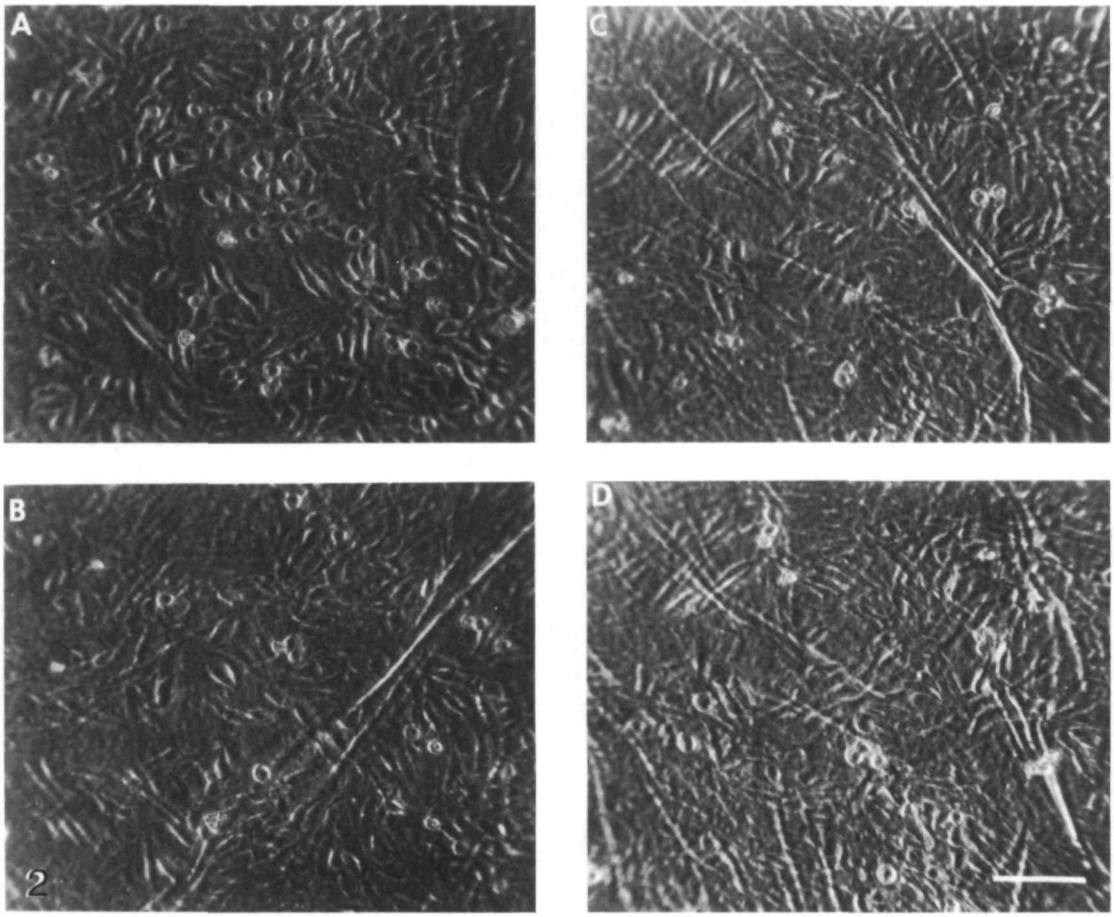

Fig. 2. Increase in contractile strength of CHO cells cultured on a silicone rubber substratum during reverse transformation by db-cAMP. The same field of view is shown at the four successive times. Bar, 100 μm. A. Time 0, introduction of db-cAMP; B, 2 h in db-cAMP; C, 3 h in db-cAMP; D, 5 h in db-cAMP.

reflexion image of the reverse transformed CHO cells closely resembles that of 3T3 cells or normal chick heart fibroblasts (Heath & Dunn, 1978, see their Fig. 3E).

When these reverse transformed CHO cells were returned to medium lacking db-cAMP and testosterone, their interference reflexion images slowly reverted back to the transformed morphology. The focal black-appearing (10 nm) contacts were progressively replaced by broader grey-appearing (30 nm) contacts. The appearance is as if the components of the black adhesions were dispersing into the white non-adherent areas so that the whole area becomes grey. We must remember, however, that these colours represent gap distances between the substratum and the lower plasma membrane of the cell – they do not represent dispersing substances *per se*. As the grey close-contacts expand gradually in area, the cell margins themselves retreat and the cells become less flattened. All of these changes became pronounced after only 3 h in the hormone-free medium and were well advanced at 12 h (Fig. 1F, G). However, even

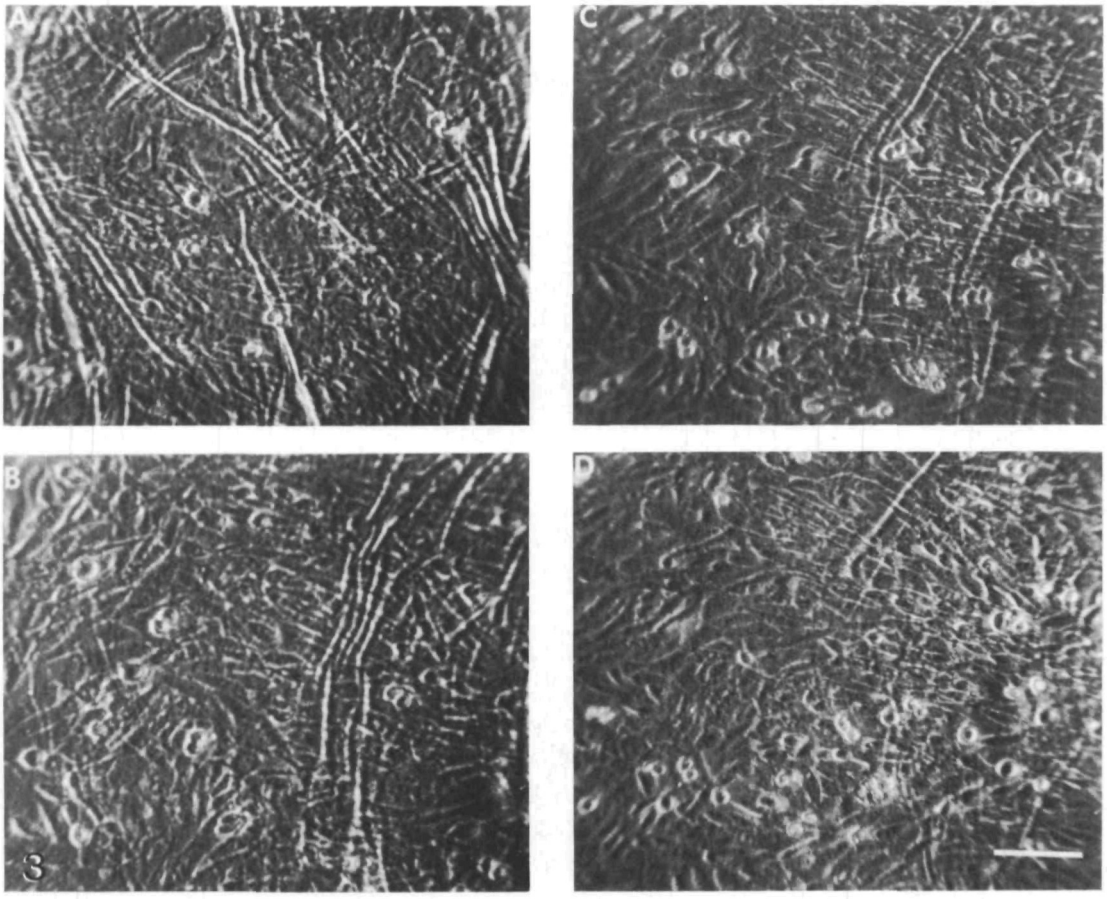

Fig. 3. Decrease in contractile strength of reverse transformed CHO cells following removal of db-cAMP. The same field of view is shown at the four successive times. Bar, 100 μm. A. Time 0, removal of db-cAMP; B, 2 h after removal of db-cAMP; C, 4 h after removal of db-cAMP; D, 6 h after removal of db-cAMP.

after 18 h the pattern of substratum contacts had not yet reverted completely to that which had existed prior to reverse transformation. This reversion was not complete until 20–25 h.

Contraction of silicone rubber substrata

While in the transformed state (before exposure to db-cAMP) the CHO cells were only weakly contractile. They were able to wrinkle the silicone rubber substrata to a slight degree only, even when cultured at confluent cell densities (see Fig. 2A). Single individual CHO cells were not sufficiently contractile to wrinkle these elastic substrata appreciably by themselves, even after reverse transformation by db-cAMP.

Following addition of db-cAMP and testosterone to their culture medium, the confluent sheets of CHO cells began to produce markedly more wrinkling of their silicone rubber substrata (see Fig. 2), indicating an increase in the contractile strength of the cells. Both the number and the size of these substratum wrinkles increased substantially within the first 2 h, and had reached an apparent plateau after 5 h in the db-cAMP-containing medium. After this time (5 h) the contractility of the cells did not appear to increase further, but remained at the same level for as long as the cells continued to be cultured in medium that contained db-cAMP and testosterone.

This increased contractility was entirely reversible, however (see Fig. 3). When returned to (conditioned) medium lacking db-cAMP and testosterone, the reverse transformed cells quickly began to relax the contractile tension they had been exerting on the rubber substratum. Re-expansion and progressive disappearance of wrinkles in the rubber substratum was apparent after only 1 h and was especially rapid and dramatic during the time between 1 h and 2 h after return to the cAMP free medium; during this particular period the elastic re-expansion of the previously crumpled rubber sheet was intermittently rapid enough to be seen directly, that is without time-lapse acceleration. This rapid re-expansion of the rubber occurs partly by the stretching of attached cells and partly by the detachment and tearing away of cells and cell clusters from this elastic substratum.

DISCUSSION

Our observations tend to support the general pattern of previous conclusions that the neoplastic transformation of cells involves reductions in both their adhesiveness and their contractility (Johnson & Pastan, 1972). Indeed our observations may even suggest a causal linkage of some sort between these two parallel deficiencies – in that the tight focal contacts that are lost in the transformed state are known from other studies to be the sites of insertion of the acto-myosin stress fibres, which are believed to be the agents of the contractility of the cells. Despite this suggestion of a causal link, it is not at all clear whether it is the loss of the tight focal contacts that causes the loss of contractility, the reverse, or whether the interaction between adhesiveness and contractility is reciprocal.

The most unexpected and difficult of our observations to explain is that the changes in contractile strength occur (or at least are completed) so much more quickly than the

changes in adhesive morphology. Notice that only 5 h were required for the CHO cells to distort the rubber substratum maximally following perfusion with db-cAMP, while the conversion from broad 30 nm contacts to focal 10 nm contacts did not reach completion for 20–25 h. The same contrast in time-course was also seen in the reversion back to the transformed state following return to normal medium. It is conceivable that differences in adhesive or other properties between silicone rubber substrata and glass may account for part of this time disparity. In other words, the adhesive changes might for some reason have actually occurred faster when the cells were cultured on rubber. The interference reflexion image afforded by the rubber layer was not sufficiently clear to reveal whether this was so, however. This possibility does not seem likely, in that the silicone rubber is, if anything, less rather than more adhesive to cells than is glass. For example, cells will move preferentially from rubber to glass whenever given a choice. So we have no reason to expect that cells would form adhesions to rubber more quickly than to glass. However, what we seem to be dealing with in this case is the conversion of one kind of surface contact into another, rather than the formation of new adhesions. We simply do not know what this conversion consists of, or how the physical properties of the substratum should affect the speed of such a conversion.

The different population densities used in the two types of observations (subconfluent in the interference reflexion observations, so that cell-to-substratum contacts would predominate over intercellular adhesions, but confluent when rubber substrata were used, because the isolated cells were insufficiently contractile by themselves to wrinkle the rubber) may possibly have been responsible for the contrast in time-courses. For example, a higher population density might somehow accelerate the development of both cellular contractility and focal adhesions.

Perhaps the simplest explanation for our results is that transformation somehow disconnects the mechanical linkages through which the cytoplasmic acto-myosin exerts its contractility upon the external substratum. The effect of reverse transformation would thus be to restore the strength of this mechanical linkage. On the other hand, the gradual conversion from broad close-contacts to narrower but closer tight contacts (as we observed during reverse transformation) seems to imply a progressive clustering together of whatever cell surface or membrane components are responsible for forming adhesions to external objects. If this 'focalization' of the adhesions does indeed reflect some sort of agglutination of adhesion molecules within the plane of the surface, then perhaps the more likely explanation for transformation itself is that the mechanical linkages that fail are lateral ones whose function is to interconnect the members of some class of surface or sub-surface components. Without these lateral links to hold them together in clusters, both the adhering materials on the exterior surface and the acto-myosin fibres just within the membrane would be free to disperse laterally. As a result, neither focal adhesions nor the stress fibres that terminate at them could be organized. Restoration of these lateral links in reverse transformed cells would permit these materials to reaggregate laterally again, thereby forming focal adhesion plaques connected to stress fibres. The likely importance of such lateral linkages between cellular components involved in adhesions has been stressed by Rees, Lloyd & Thom (1977).

We have previously shown that the strong traction force exerted by untransformed fibroblasts and other cells has the effect of rearranging adjacent collagenous matrices to form structures much like organ capsules, and we have proposed that this 'tractional restructuring' is the normal mechanism by which capsules are formed (Harris, Stopak & Wild, 1981). As it happens, one of the characteristic differences between benign and malignant tumours is that the former become surrounded by capsules while the latter do not. We suggest that it is the weakened tractional strength of malignant cells that accounts for this failure to form capsules.

We greatly appreciate the expert advice and help provided by Ms Mary Anne Pendergast. We thank Dr Arthur Lockwood for the gift of the CHO cells and testosterone, and for several helpful discussions. We are also greatly indebted to Ms Patricia Warner for her excellent technical assistance.

REFERENCES

Bloom, G. S. & Lockwood, A. H. (1980). Redistribution of myosin during morsiveness, a property of cells from squamous cell carcinomas. *Cancer Res.* **4**, 625–629.

Carter, S. B. (1968). Tissue homeostasis and the biological basis of cancer. *Nature, Lond.* **220**, 970–974.

Coman, D. R. (1944). Decreased mutual adhesiveness, a property of cells from squamous cell carcinomas. *Cancer Res.* **4**, 625–629.

Curtis, A. S. G. (1964). The mechanism of adhesion of cells to glass. A study by interference reflexion microscopy. *J. Cell Biol.* **20**, 199–210.

Gershman, H., Drumm, J. & Culp, L. (1976). Sorting out of normal and virus-transformed cells in cellular aggregates. *J. Cell Biol.* **68**, 276–286.

Gompel, C. (1978). *An Atlas of Diagnostic Cytology*. New York: Wiley.

Harris, A. K. (1973*a*). Location of cellular adhesions to solid substrata. *Devl Biol.* **35**, 97–114.

Harris, A. K. (1973*b*). Cell surface movements related to cell locomotion. *Ciba Fdn Symp. 14, Locomotion of Tissue Cells* (ed. R. Porter & D. W. Fitzsimons), pp. 1–26. Amsterdam: Elsevier.

Harris, A. K., Stopak, D. & Wild, P. (1981). Fibroblast traction as a mechanism of collagen morphogenesis. *Nature, Lond.* **290**, 249–251.

Harris, A. K., Wild, P. & Stopak, D. (1980). Silicone rubber substrata: A new wrinkle in the study of cell locomotion. *Science, N.Y.* **208**, 177–179.

Heath, J. P. & Dunn, G. A. (1978). Cell to substratum contacts of chick fibroblasts and their relation to the microfilament system. A correlated interference reflexion and high voltage electron microscopic study. *J. Cell Sci.* **29**, 197–212.

Hsie, A. W. & Puck, T. T. (1971). Morphological transformation of Chinese hamster cells by dibutyryl adenosine cyclic 3′:5′-monophosphate and testosterone. *Proc. natn. Acad. Sci. U.S.A.* **68**, 358–361.

Izzard, C. S. & Lochner, L. R. (1976). Cell-to-substratum contacts in living fibroblasts: an interference reflexion study with an evaluation of the technique. *J. Cell Sci.* **21**, 129–160.

Johnson, G. S., Friedman, R. M. & Pastan, I. (1971). Restoration of several morphological characteristics of normal fibroblasts in sarcoma cells treated with adenosine 3′:5′-cyclic monophosphate and its derivatives. *Proc. natn. Acad. Sci. U.S.A.* **68**, 425–429.

Johnson, G. S. & Pastan, I. (1972). Cyclic AMP increases the adhesion of fibroblasts to substratum. *Nature, new Biol.* **236**, 247–249.

Porter, K. R., Puck, T. T., Hsie, A. W. & Kelly, D. (1974). An electron microscope study of the effects of dibutyryl cyclic AMP on Chinese hamster ovary cells. *Cell* **2**, 145–162.

Puck, T. T., Waldren, C. A. & Hsie, A. W. (1972). Membrane dynamics in the action of dibutyryl adenosine 3′:5′-cyclic monophosphate and testosterone on mammalian cells. *Proc. natn. Acad. Sci. U.S.A.* **69**, 1943–1947.

REES, D. A., LLOYD, C. W. & THOM, D. (1977). Grip control and stick in cell adhesion through lateral relationships of membrane glycoproteins. *Nature, Lond.* **267**, 124–127.

STEINBERG, B. M., SMITH, K., COLOZZO, M. & POLLACK, R. (1980). Establishment and transformation diminish the ability of fibroblasts to contract a native collagen gel. *J. Cell Biol.* **87**, 304–308.

VASILIEV, J. M. & GELFAND, I. M. (1981). *Neoplastic and Normal Cells in Culture*. Cambridge University Press.

(Received 10 March 1983 – Accepted 12 May 1983)